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TURPENTINE-INDUCED DECREASE OF α_1-FOETOPROTEIN IN THE SERUM OF THE DEVELOPING RAT : A NOVEL PARAMETER OF THE INFLAMMATORY RESPONSE

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The acute phase of an inflammation induced in young rats, at various ages between 1 and 21 days after birth, by a single subcutaneous turpentine injection, is accompanied by a 35%-45% decrease of the α_1-foetoprotein serum concentrations. This fall is demonstrated with equilibrium dialysis binding methods, evidencing the loss of about 1/3 of the high affinity estrogen binding sites characteristic for the rat foetoprotein, as well as by specific immunoassay of the foetoprotein concentrations. This negative response is coincidental with classical inflammatory changes of the plasma proteins, similar to those defined in adults : in particular we evidence 5 to 10 fold rises of haptoglobin, as early as the first post-natal day. We discuss the implications of these findings for ontogenic and mechanistic aspects of the inflammatory response.

We report that the estrophilic α_1-foetoprotein (AFP) of the immature post-natal rat reacts to the inflammatory injury which follows a subcutaneous turpentine injection by a marked decrease of its serum concentration. Therefore AFP behaves in the growing rat as an acute phase reactant (APR) with a negative response : this represents a novel feature of the plasma inflammatory response and constitutes the first report on α_1-foetoprotein as a marker of inflammation.

We remind that AFP is a largely spread carcinofoetal antigen (1) which only in the rat persists for about 23 days after birth, i.e. throughout the post-natal maturation (2). Rat AFP shares only with the mouse foetoprotein the ability to bind closely the estrogen hormones (3,4). A further singular characteristic of the murine AFP is the competitive inhibition of its interaction with the estrogens by nonsteroid ligands, namely polyunsaturated nonesterified fatty acids, including arachidonate (5,6).

The role of rat AFP as a negative APR will be discussed in relation to its unique ontogenic and binding properties.

MATERIAL AND METHODS

Animals, injections and sera. Rats of the Sprague-Dawley CD strain (Charles River, France) were used in all experiments. Neonates were housed with their mothers (10 pups per dam) under controlled conditions of light (14 h. light/day) and temperature (22-23° C). The animals were allowed free access to water and rat laboratory chow.

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The inflammatory reaction was induced by a single subcutaneous injection of 3 µl turpentine oil/g animal, i.e. 50% of the lethal dose for immatures, established as a preliminary. A local inflammation was observed in all the treated pups. Injections were given at the same hour (6 pm). One half of each litter received turpentine while the other half (controls) received an injection of physiological saline. It was assessed that saline-treated or intact rats of the same age displayed identical serum parameters.

Blood was collected under brief (less than 1 min.) ether anesthesia by axillary puncture, 40 h. after the injections. According to previous studies (7) or preliminary tests, this interval allowed maximum responses of the classical APRs haptoglobin and orosomucoïd, as well as of the novel paremeters under study. Pooled sera from experimental and control littermates were prepared by centrifugation at 4000 rpm and 4°C.

Purified proteins, antisera and hormones. Rat AFP was purified to electrophoretic and immunological homogeneity by preparative gel electrophoresis, as described (8). Specific anti-AFP antisera were raised in the rabbit. Purified rat haptoglobin and monospecific anti-haptoglobin rabbit antisera were a kind gift from Professor R. Engler.

2,4,6,7 (n)-{^{3}H}estradiol-17β, (E_2) 87 Ci/mmol, was from the Radiochemical Center, Amersham, and unlabelled E_2 from Roussel Uclaf.

Binding studies. The batchwise gel equilibration technique of Pearlman and Crepy, using a suspension of Sephadex G-25 as the semi-permeable membrane (9), Scatchard analysis (10) and Rosenthal correction (11) of binding data were applied. Endogenous steroids were removed by charcoal treatment (12). Detailed descriptions and assessments of these methods have been given elsewhere (6,13).

The following parameters were determined : 1/P binding indices (L/g), expressing the general ability of a protein mixture to bind a ligand, where P is the protein concentration corresponding to an equilibrium ratio of bound/free steroid = 1 ; association constants (Ka), (M^{-1}) ; maximum number of binding sites/g of serum proteins ($n_1M_1 x\ g^{-1}$).

Total and specific protein determinations. Total serum proteins were measured by the colorimetric method of Lowry et al. (14). For the specific assay of the serum AFP and the serum haptoglobin we have used the electroimmunodiffusion technique of Laurell (15).

RESULTS

Effect of the turpentine-induced injury on the estradiol binding activity of the immature rat.

AFP is responsible for virtually the entire E_2 binding ability of immature rat sera : therefore the level of E_2 binding by whole sera provides a reliable indication of the concentration and/or functionnal status of the foetoprotein.

Table 1 compares the E_2 binding indices 1/P (L/g) in sera from inflammatory and control rats, 40 h. after a s.c. injection of turpentine oil or physiological saline respectively. It may be seen that in all the studied age groups the turpentine treatment causes a marked decrease - 35%-45% - of the estrogen binding.

These results point to an inflammatory-induced decrease of the serum concentration or of the binding activities of the AFP.

TABLE 1

"1/P" estradiol binding indices (L/g) of sera from inflammatory and control rats. Means of determinations on 4 serum pools ± SE.

Age at injection (days, post-natal)	Inflammatory (turpentine-treated)	Controls (saline-treated)	% of control binding
1	47,7 ± 31,05 *	72,9 ± 37,2	63,6 ± 16,29
4	29,25± 14,8 *	51,25± 15,6	57,17± 19,9
10	18 ± 5,2 *	30,5 ± 9,5	60,19± 11,11
21	6,05± 0,34 *	9,15± 1,3	60,18± 2,68

* Statistically significant compared with controls P<0,001 by Student's t test.

Effect of the turpentine - induced injury on the thermodynamic equilibrium parameters of the estradiol-serum AFP interaction : Scatchard analysis.

All the high affinity E_2 binding sites of the immature rat serum are carried by AFP (3,4) ; their equilibrium binding constants can be accurately determined by Scatchard analysis of the interactions between E_2 and whole sera, if a graphic correction is applied for the interference of the nonspecific low affinity E_2 binding sites, contributed mainly by albumin.

In Table 2 and Fig. 1 we present the association constants (Ka_1) and apparent concentrations (n_1M_1/g serum proteins) thus determined for the E_2 sites of AFP in inflammatory and control sera.

The results clearly show that the turpentine treatment does not affect the Ka_1 values, which measure the affinity of the binding interaction, but lessen significantly - by about 35-45% - the $n_1M_1\ g^{-1}$ capacity parameters. This fall of n_1M_1 concentrations is in fair agreement with that of the " 1/P " E_2 binding indices described above.

We conclude that the turpentine injection brings about the partial serum depletion of AFP estrophilic sites, rather than an impairment of their affinity characteristics.

TABLE 2

Association constants (Ka_1) and maximum number of binding sites/g serum proteins ($n_1M_1g^{-1}$) for the interactions of E_2 with whole sera from inflammatory and control rats. Means of determinations on 3 different serum pools ± SE.

Age at injection (days)	Inflammatory (Turpentine-treated)		Controls (Saline-treated)	
	$Ka_1 x10^8 M^{-1}$	$n_1M_1g^{-1}x10^{-7}$	$Ka_1 x10^8 M^{-1}$	$n_1M_1g^{-1}x10^{-7}$
4	0,82 ± 0,01*	4,4 ± 0,03**	0,84 ± 0,02	6,6 ± 0,02
21	1,1 ± 0,01*	0,62± 0,02**	1,1 ± 0,02	0,98± 0,03

* Difference from controls : Non significant.
** Difference from controls : significant to P<0,001 by Student's t test.

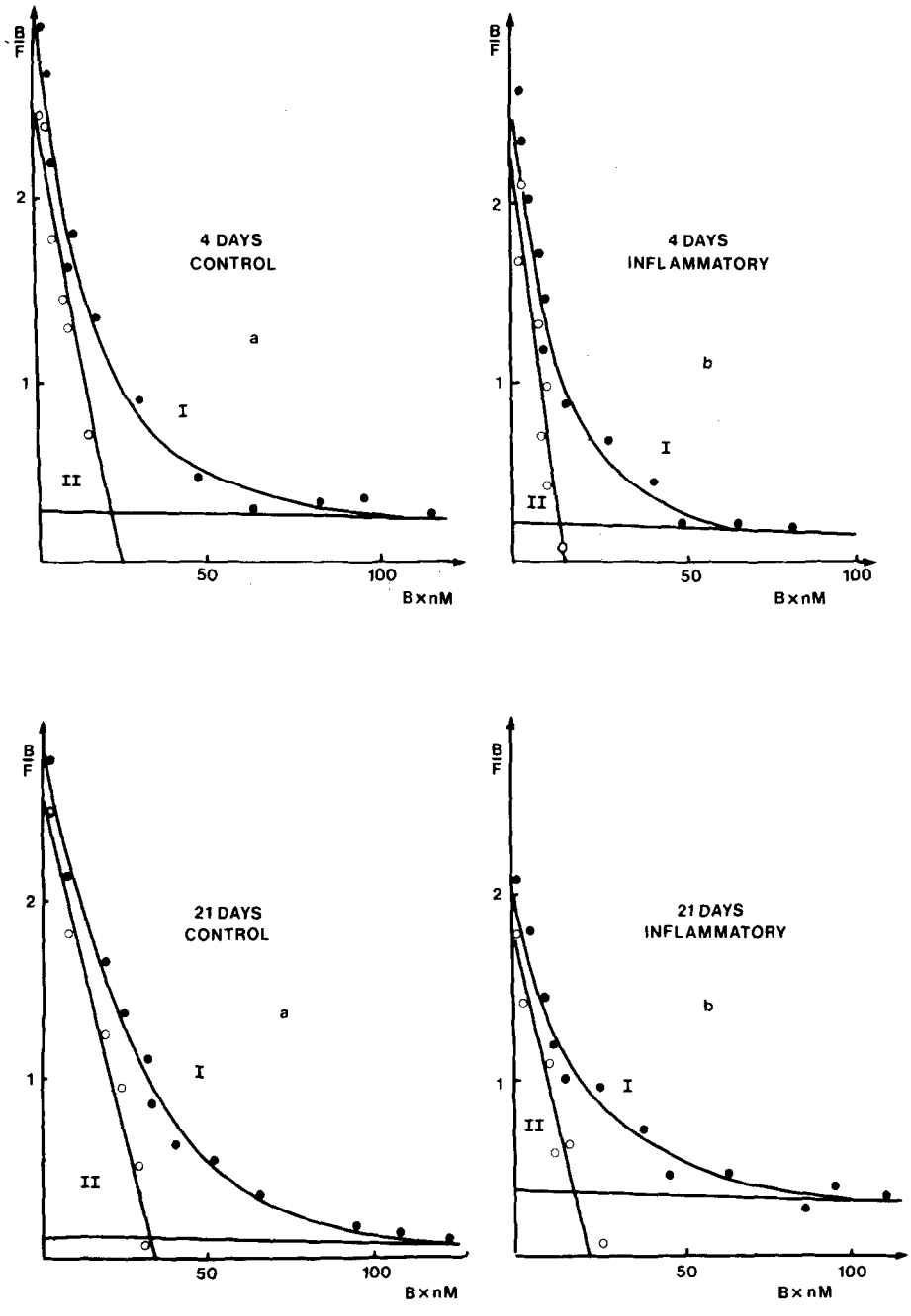

Fig. 1 : Scatchard plots (Curves I) and Rosenthal corrections (curves II) for the binding of E_2 to control (a) and inflammatory (b) rats, treated at 4 and 21 days. Reaction mixtures consisted of : 200 mg Sephadex G-25 (fine), 70, 78, 350 and 360 µg serum proteins (4 days a & b, and 21 days a & b respectively), 1-600 ng E_2, in 2 ml of 0,15 M PO_4 H Na_2 /PO_4 H_2 K buffer, pH 7.4. Each point is the mean of a triplicate assay.
4 days : Ka = 0,84 and 0,82 10^8 M^{-1} for control and inflammatory, n_1M_1 g^{-1} = 6,6 and 4,4 10^{-7} M for control and inflammatory.
21 days : Ka = 1,1 and 1,2 10^8 M^{-1} ; n_1M_1 x g^1 = 0,98 and 0,62 10^{-7} M for control and inflammatory respectively.

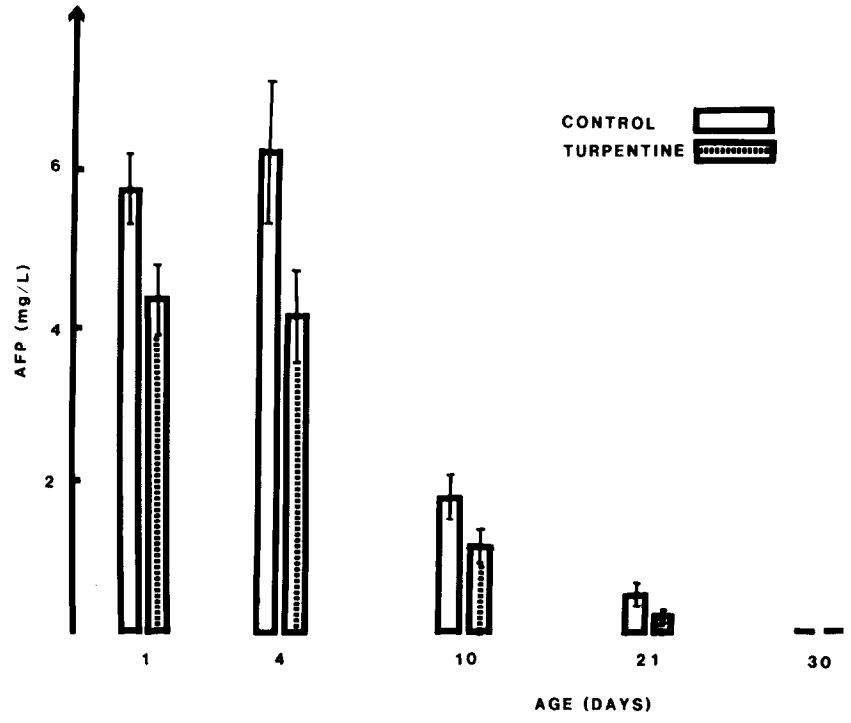

Fig. 2 : AFP concentrations in sera from control and inflammatory post-natal rats. The determinations were carried out with the electroimmunodiffusion technique of Laurell, using monospecific rabbit anti-AFP antisera against highly purified rat AFP. Means of 4 determinations on separate pools ± SE.

Effect of the turpentine-induced injury on the serum AFP concentrations : immunological studies.

The decreased levels of E_2 binding sites after the turpentine injection could result either from an actual loss of the serum foetoprotein, or from the partial masking of its estrophilic sites due to possible inflammation-related inhibitory factors. To clarify this point we have carried out the specific immunological assay of AFP in the inflammatory and control sera.

The results are illustrated in Fig. 2. It may be seen that the AFP concentrations (mg/ml serum) are decreased by about 35-45% in the turpentine-treated rats versus their physiological saline-treated littermates, in all the age groups under study. Thus we unambiguously demonstrate that the inflammatory decrease of the serum E_2 binding capacity results from an actual loss of the AFP and that the relative magnitude of this response is maintained throughout development.

Effects of the turpentine-induced injury on the haptoglobin levels of immature rat sera.

To our knowledge, no data are available concerning the inflammatory plasma response of the perinatal rat. In order to definitively class the AFP of the turpentine-injured immature rat with the acute phase proteins, it seemed desi-

TABLE 3

Haptoglobin concentrations (μmol/L) in inflammatory and control sera from developing and adult rats. Means of determinations on 4 serum pools ± SE.

Age at injection (days)	Inflammatory (turpentine-treated)	Controls (saline-treated)
1	4 ± 3,1	0,45 ± 0,09
4	13,5± 2,1	0,6 ± 0,25
10	22,5± 3	1,5 ± 0,6
21	37,5± 4	6,9 ± 2,5
60	18 ± 2	1,9 ± 0,1

At all ages differences from controls are statistically significant to P<0,001 (Student's t test).

rable to ascertain that its decrease is implied in an overall reaction at least partly analogous to that described for the adults. A detailed study on the markers of inflammation during development is currently completed in our laboratory and will be reported subsquently.

We show here as an example (table 3) how the turpentine injections affect in the growing rats the serum levels of haptoglobin (Hp), a positive APR extensively studied in the adult (16).

Striking inflammatory Hp increases are seen as early as one day of age. This effect appears even higher than in adults for the animals aged 4 days or 10 days at the time of the experiment.

Our date demonstrate the inflammatory competence of the developing rat. They also point to complex age-dependent features of the inflammatory reaction, whether the differences between the immatures and the adults be of degree, as in the haptoglobin instance, or of nature, as in the case of the response of AFP, highly specific for the immatures.

CONCLUSIONS AND DISCUSSION

We demonstrate that the injury of subcutaneously injecty turpentine in post-natal developing rats, at various ages between 1 and 21 days, causes the loss of about one-third of the highly oestrophilic serum α_1-foetoprotein. Consequently, about one-third of the high affinity estrogen binding sites also disappear from the sera. The AFP decrease coincides with known inflammatory effects, e.g. important rises of haptoglobin, a classical acute phase reactant described in the adults. These findings allow to reckon the rat AFP among the markers of inflammation, more precisely among the acute phase reactants with a negative response - or " negative " APRs -.

The decrease of AFP observed in the turpentine-treated neonatal and infantile rats contrasts both to its lack of response in the foetus in utero whose

mother undergoes experimental inflammation (17) and to its reappearance and rise in the adult during severe hepatic disorders, like dimethylaminoazo-benzene induced hepatomas (18) or C Cl_4 intoxications (19). The opposite variations of the rat AFP as an inflammation marker and as a tumor maker deserve special emphasis, since confusion frequently persists between these two types of indicators (16,20). The inflammatory AFP decrease is in agreement with the suppressed serum AFP levels observed in newborn rats after administration of glucocorticoids (19). Indeed the inflammatory reaction is accompanied by important rises of the endogenous corticosteroids (7,24), and this phenomenon could have effects similar to those exerted by the injected glucocorticoids. Detailed metabolic studies are necessary to clarify these mechanisms ; in particular it remains to determine whether the turpentine-induced AFP decrease implies inhibition of the protein synthesis, increase of its catabolism, transfer to extravascular spaces or inhibition of its secretion.

Without predjudging such mechanisms, an evaluation may be already attempted of the physiological significance of the inflammatory AFP decrease. The murine foetoprotein binds closely two classes of dissimilar compounds, the estrogen hormones and the nonsteroid polyenic fatty acids (3,6). The inflammatory-induced fall of the carrier protein will increase the unbound portions of both types of ligands and thus strengthen their physiological impacts. In addition, it should be noted that the biochemical markers of inflammation are thought to be involved in beneficial anti-inflammatory mechanisms (21,22). In the light of these considerations, the " activation " of estrogens and fatty acids through their release from AFP suggests their participation in definite steps of an overall defensive reaction.

The points of impact of the fatty acids could be sought in the metabolic pathways of the prostaglandin and the hydroxy fatty acid syntheses from arachidonate. The hydroxy fatty acids have been recognized as active contributors to the immune response, while the prostaglandins are well studied key factors in the cascade of events of the inflammatory process (22). The AFP response, by modulating the availability of the fatty acid precursor might play a role in the production and activities of these important derivatives.

As to the estrogens, the search for a regulatory role in the synthesis of the " positive " APRs might prove fruitful. In this respect, it is suggestive to bring together the following findings : the thyroxine and the glucocorticoids are among the hormonal factors implicated in the control of the haptoglobin synthesis (23) ; on the other hand, the specific protein carriers of these hormones, i.e. the thyroxin binding prealbumin and the transcortin, decrease in the serum of the inflammatory rat (17,20). One of the functions of the " negative " APRs could be to liberate the hormones (or other small molecules) that trigger or modulate essential steps of the inflammatory reaction, in particular the hepatic synthesis of the " positive " reactants.

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